CLAIMS

What is claimed is:

1. An isolated nucleic acid fragment encoding a nitrilase enzyme selected from the group consisting of:

(a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:14;

(b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:14;

(c) an isolated nucleic acid molecule that hybridizes with the isolated nucleic acid fragment of (a) under hybridization conditions of 6X SSC (1M NaCl), 40 to 45 % formamide, 1 % SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C; and

(d) an isolated nucleic acid fragment that is completely complementary to (a), (b) or (c).

2. An isolated nucleic acid fragment comprising a first nucleotide sequence encoding a polypeptide of at least 369 amino acids that has greater than 71 % identity based on the Needleman and Wunsch algorithm when compared to a polypeptide encoded by the sequence identified in SEQ ID NO:5, or a second nucleotide sequence comprising the complement of the first nucleotide sequence.

3. An isolated nucleic acid fragment encoding a nitrilase enzyme, or a fragment thereof, selected from the group consisting of:

(a) an isolated nucleic acid fragment selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15 and SEQ ID NO:16;

(b) an isolated nucleic acid molecule that hybridizes with the isolated nucleic acid fragment of (a) under hybridization conditions of 6X SSC (1M NaCl), 40 to 45 % formamide, 1 % SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C; and

(c) an isolated nucleic acid fragment that is completely complementary to (a) or (b).

4. An isolated nucleic acid sequence encoding a nitrilase enzyme selected from the group consisting of SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:16.

5. The isolated nucleic acid fragment of any of Claims 1, 2, 3, or 4 wherein the fragment is isolated from an *Acidovorax* strain.

6. A polypeptide encoded by the nucleic acid fragments of any of Claims 1, 2, 3, or 4.

7. A polypeptide according to Claim 6 having the amino acid sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:14.

8. The polypeptide of Claim 6 further characterized by nitrilase activity on nitrile-containing substrates selected from the group consisting of aliphatic nitriles and aromatic nitriles.

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9. A chimeric gene comprising the isolated nucleic acid fragment of any of Claims 1, 2, 3, 4 or 5 operably linked to suitable regulatory sequences.

10. A plasmid pSW91 contained in *E. coli* SW91 having the designation ATCC PTA-1175, a plasmid pnit4 contained in *E. coli* DH5 α: pnit4 having the designation ATCC PTA-1176, or a plasmid pnitex2 contained in either *E. coli* SS1002 or in *E. coli* SS1011.

11. An expression cassette comprising the chimeric gene of Claim 9.

12. The expression cassette of Claim 11 selected from the group consisting of the plasmids pSW91, pnit4, and pnitex2.

13. A transformed microorganism comprising the chimeric gene Claim 9.

14. A transformed microorganism comprising the plasmid of Claim 10.

15. A transformed microorganism comprising the expression cassette of Claim 11.

16. The transformed microorganism of Claim 15 wherein the expression cassette is chromosomally integrated.

17. The transformed microorganism of Claim 16 further comprising suitable regulatory sequences.

18. The transformed microorganism of Claim 17 wherein the suitable regulatory sequences comprise

a) at least one promoter selected from the group consisting of the tryptophan operon promoter Ptrp of *E. coli*, a lactose operon promoter Plac of *E. coli*, a Ptac promoter of *E. coli*, a phage lambda right promoter PR, a phage lambda left promoter PL, a T7 promoter, a promoter of the AOX1 gene from *Pichia pastoris*, and a promoter of the GAP gene from *Pichia pastoris,* or is at least one strong promoter selected from the group consisting of *Comamonas*, *Corynebacterium*,

Brevibacterium, Rhodococcus, Azotobacter, Citrobacter, Enterobacter, Clostridium, Klebsiella, Salmonella, Lactobacillus, Aspergillus, Saccharomyces, Pichia, Zygosaccharomyces, Kluyveromyces, Candida, Hansenula, Dunaliella, Debaryomyces, Mucor, Torulopsis, Methylobacteria, Bacillus, Escherichia, Pseudomonas, Rhizobium, and *Streptomyces*, and

b) at least one ribosome binding site from a phage lambda CII gene or selected from the group consisting of ribosome binding sites from a gene of *Comamonas, Corynebacterium, Brevibacterium, Rhodococcus, Azotobacter , Citrobacter, Enterobacter, Clostridium, Klebsiella, Salmonella, Lactobacillus, Aspergillus, Saccharomyces, Zygosaccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Dunaliella, Debaryomyces, Mucor, Torulopsis, Methylobacteria, Bacillus, Escherichia, Pseudomonas, Rhizobium*, and *Streptomyces.*

19. The transformed microorganism of Claim 18, wherein the host microorganism is selected from the group consisting of *Comamonas, Corynebacterium, Brevibacterium, Rhodococcus, Azotobacter, Citrobacter, Enterobacter, Clostridium, Klebsiella, Salmonella, Lactobacillus, Aspergillus, Saccharomyces, Zygosaccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Dunaliella, Debaryomyces, Mucor, Torulopsis, Methylobacteria, Bacillus, Escherichia, Pseudomonas, Rhizobium*, and *Streptomyces.*

20. A transformed microorganism selected from the group consisting of:

(a) *E. coli* SW91 having the designation ATCC PTA-1175;

(b) *E. coli* DH5α: pnit4 having the designation ATCC PTA-1176;

(c) *E. coli* SS1001 having the designation ATCC PTA-1177; and

(d) *E. coli* SS1002 containing plasmid pnitex2; and

(e) *E. coli* SS1011 containing plasmid pnitex2.

21. A method of obtaining a nucleic acid fragment encoding all or a substantial portion of a nitrilase enzyme, the method comprising:

(a) probing a genomic library with all or a portion of a nucleic acid fragment selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NIO:13, SEQ ID NO:15, and SEQ ID NO:16;

(b) identifying a DNA clone that hybridizes with the nucleic acid fragment of step (a); and

(c) sequencing the nucleic acid fragment that comprises the DNA clone identified in step (b),

wherein the sequenced nucleic acid fragment of step (b) encodes all or a substantial portion of an amino acid sequence encoding a nitrilase enzyme.

22. A method of obtaining a nucleic acid fragment encoding all or a substantial portion of a nitrilase enzyme, the method comprising:

(a) synthesizing at least one oligonucleotide primer corresponding to a portion of the sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:16; and

(b) amplifying an insert present in a cloning vector using the oligonucleotide primer of step (a), the amplified insert of step (b) encoding all or a substantial portion of an amino acid sequence encoding a nitrilase enzyme.

23. The product of the method of Claims 21 or 22.

24. A method to enzymatically convert nitrile-containing substrates to a carboxylic acid, the method comprising:

(a) contacting, under suitable conditions, a transformed heterologous host expressing the polypeptide of Claim 4 with a nitrile-containing substrate; and

(b) optionally collecting the carboxylic acid produced in step (a).

25. The method of Claim 24, wherein the nitrile-containing substrate is a dinitrile of the formula NC-R-CN where R is an alkylene group having from 1 to 10 carbon atoms.

26. The method of Claim 25, wherein the nitrile-containing substrate is 2-methylglutaronitrile.

27. A method to enzymatically convert nitrile-containing substrate(s) to carboxylic acid(s), the method comprising:

(a) contacting, under suitable conditions, a transformed heterologous host comprising the chimeric gene of Claim 9 with nitrile-containing substrate(s); and

(b) optionally collecting the carboxylic acid produced in step (a).

28. The method of Claim 27, wherein the nitrile-containing substrate is a dinitrile of the formula NC-R-CN where R is an alkylene group having from 1 to 10 carbon atoms.

29. The method of Claim 27, wherein the nitrile-containing substrate is 2-methylglutaronitrile.

30. The method of Claim 27 wherein the suitable regulatory sequences of the chimeric gene comprise an inducible promoter.

31. The method of Claim 30, the suitable conditions of step (a) further comprising the presence of an inducer of the inducible promoter.

32. The method of enzymatically converting 2-methylglutaronitrile to the corresponding carboxylic acid, the method comprising:

(a) contacting, under suitable conditions, *E. coli* SW91 designated ATCC PTA-1175 with 2-methylglutaronitrile; and

(b) optionally collecting the carboxylic acid produced in step (a).

33. An improvement to the process for preparing five-membered ring lactams or six-membered ring lactams from aliphatic α,ω-dinitriles comprising:

(a) contacting an aliphatic α,ω-dinitrile in an aqueous reaction mixture with an enzyme catalyst, whereby the aliphatic α,ω-dinitrile is converted to an ω-cyanocarboxylic acid ammonium salt;

(b) contacting the aqueous product mixture resulting from step (a) with hydrogen and a hydrogenation catalyst, whereby the ω-cyanocarboxylic acid ammonium salt is converted directly to the corresponding lactam without isolation of the intermediate ω-cyanocarboxylic acid, ω-cyanocarboxylic acid ammonium salt, ω-aminocarboxylic acid, or ω-aminocarboxylic acid ammonium salt; and

(c) recovering the lactam from the aqueous product mixture resulting from step (b);

the improvement comprising in step (a) contacting an aliphatic α,ω-dinitrile in an aqueous reaction mixture with an enzyme catalyst selected from the group consisting of

(1) *E. coli* SW91 having the designation ATCC PTA-1175;

(2) *E. coli* DH5a: pnit4 having the designation ATCC PTA-1176;

(3) *E. coli* SS1001 having the designation ATCC PTA-1177; and

(4) *E. coli* SS1002 containing plasmid pnitex2, and

(5) *E. coli* SS1011 containing plasmid pnitex2.

34. The process of Claim 33 wherein the aliphatic α,ω-dinitrile has the formula

$$NCCX_a(R)(CH_2)_nCN,$$

where a = 0 or 1, where X = hydrogen when a = 1, and R = H, alkyl or substituted alkyl, or alkenyl or substituted alkenyl, or alkylidene or substituted alkylidene, and where n = 1 or 2.

35. The method of Claim 34 wherein the α,ω-dinitrile is 2-methylglutaronitrile.

36. The process of Claim 33 wherein the aliphatic α,ω-cyano is unsymmetrically substituted at the α-carbon atom, and the enzyme catalyst is characterized by aliphatic nitrilase activity that produces the ω-cyanocarboxylic acid ammonium salt resulting from regioselective hydrolysis of the ω-cyano group, thereby producing only one of the two possible lactam products during step (b).

37. The process of Claim 33 further comprising adding, before step (b), ammonium hydroxide, ammonia gas, or methylamine to the aqueous product mixture containing the ω-cyanocarboxylic acid ammonium salt

38. The process of Claim 37 wherein the amount of ammonium hydroxide, ammonia gas, or methylamine added to the aqueous product mixture is from 0 to 4 molar equivalents relative to the amount of ω-cyanocarboxylic acid ammonium salt present.

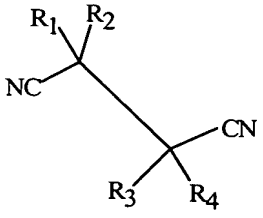
39. The process of Claim 38 wherein methylamine is added to the aqueous product mixture containing the ω-cyanocarboxylic acid ammonium salt before step (b) and the product of step (b) is an N-methyllactam.

40. The process of Claim 33 wherein in step (b) the temperature of the aqueous product mixture is from 45 °C to 200 °C.

41. The process of Claim 33 for preparing five-membered ring lactams or six-membered ring lactams from aliphatic α,ω-dinitriles, wherein:

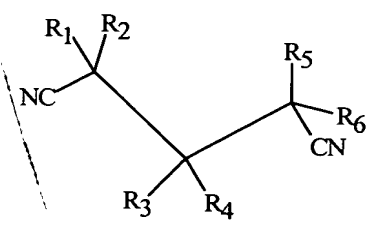
the aliphatic α,ω-dinitrile is of either formula:

I



or

II



where R_1 and R_2 are both H, and

R_3, R_4, R_5, and R_6 are each independently selected from the group consisting of H, alkyl or substituted alkyl, or alkenyl or substituted alkenyl, or R_3 and R_4 taken together are alkylidene

or substituted alkylidene, or independently R_5 and R_6 taken together are alkylidene or substituted alkylidene.

42. The method of Claim 33 or 41 wherein the enzyme catalyst is in the form of whole microbial cells immobilized in or on an insoluble support.

43. A method for using a native microbial gene encoding a protein characterized by a nitrilase activity on nitrilase-containing substrates to obtain a mutated microbial gene encoding a protein characterized by an increased specific nitrilase activity on nitrile-containing substrates and/or an increased stability of the nitrilase, one or both characteristics increased relative to that of the native microbial gene, the method comprising the steps of

(i) contacting restriction endonucleases with a mixture of nucleotide sequences to yield a mixture of restriction fragments, the mixture of nucleotide sequences comprising

a) a native microbial gene;

b) a first population of nucleotide fragments which will hybridize with the nucleotide sequences of the native microbial gene of (i)(a); and

c) a second population of nucleotide fragments which will not hybridize to the nucleotide sequences of the native microbial gene of (i)(a),

(ii) denaturing the mixture of restriction fragments of step (i);

(iii) incubating the denatured mixture of restriction fragments of step (ii) with a polymerase; and

(iv) repeating steps (i), (ii), and (iii) a sufficient number of times to yield a mutated microbial gene encoding a protein characterized by an increased specific nitrilase activity on nitrile-containing substrates and/or an increased stability of the nitrilase, one or both characteristics increased relative to the nitrilase activity of the native microbial gene.

44. The method of Claim 43 wherein the native microbial gene is *Acidovorax facilis* 72W and the nitrile-containing substrate is 2-methylglutaronitrile.

45. A mutated microbial gene encoding a protein characterized by an increased specific nitrilase activity on nitrile-containing substrates and/or an increased stability of the nitrilase, one or both characteristics increased relative to the nitrilase activity of a native microbial gene, the mutated microbial gene produced by the method of Claim 43.

46. The transformed microorganism of Claim 19, wherein the host microorganism is *E. coli* strains MG1655 (ATCC 47076), W3110 (ATCC 27325), MC4100 (ATCC 35695), or W1485 (ATCC 12435).

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